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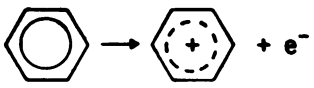
Structure–Activity Relationships in the Free-Radical Metabolism of Xenobiotics

by Colin F. Chignell*

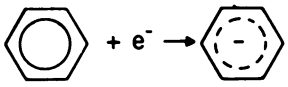
Many xenobiotics, including naturally occurring compounds, drugs, and environmental agents, are metabolized both *in vivo* and *in vitro* to free-radical intermediates. The one-electron reduction of nitroaromatic compounds, quinones, and a wide variety of other chemicals is catalyzed enzymatically by a number of reductases and dehydrogenases. Structure–activity studies have shown that the cytotoxicities of nitroaromatic compounds and quinones are related to their one-electron reduction potentials (E_7^1). Other factors such as oil:water partition coefficients may also be important. Xenobiotics may also be oxidized to free radicals by peroxidases and oxidases. Hammett's rules apply to the one-electron oxidation of simple *meta*- or *para*-substituted phenols and amines by horseradish peroxidase, compound I.

Introduction

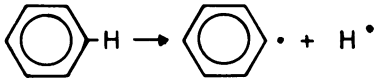
A free radical may be defined as any molecule that has an odd number of electrons (*1*). While the vast majority of known free radicals are derived from organic molecules, many inorganic radicals, e.g. $O_2^{\cdot-}$, $SO_3^{\cdot-}$, are known to exist. The transformation of an organic molecule into a free radical may be accomplished in three different ways (*2*). For example, the one-electron oxidation of benzene (by removal of one of the π-electrons) produces the benzene cation radical:



The addition of an electron to a benzene π-orbital generates the benzene anion radical:



When the C—H bond of benzene is cleaved homolytically, a hydrogen atom and the neutral phenyl radical are formed:



While benzene itself is not readily converted into a free radical, many other organic molecules do generate radicals, even under the relatively mild conditions encountered *in vivo*. To date more than twenty different classes of compounds are known to be metabolized in biological systems to free-radical intermediates (*2–4*). These chemicals include naturally occurring compounds, drugs, and environmental agents.

Detection of Free Radicals

Free radicals may be detected by using the technique of electron spin resonance (ESR). An ESR spectrum gives information on both the structure and concentration of a free radical. For example, the ESR spectrum in Figure 1 was obtained by incubating the herbicide paraquat with rat hepatic microsomes in the presence of the cofactor NADPH under anaerobic conditions (*5*). The radical derived from paraquat is readily identified from its ESR spectrum as the paraquat monocation radical based on the complex pattern of lines which arise from the interaction (hyperfine coupling) of the unpaired electron with the protons present in the molecule. The herbicidal activity of paraquat results from the reaction of its cation radical with oxygen to form superoxide, which initiates a series of events that eventually lead to lipid peroxidation (*6*). The mechanism of paraquat poisoning in man and other mammals is also thought to be superoxide-mediated, although the role of lipid peroxidation has not been unequivocally established (*7*).

Not all free radicals are stable enough to be detected directly by ESR. For such radicals, a technique called "spin trapping" may be employed (*8*). Spin trapping is a technique that involves the addition of a reactive free radical (R·) to an organic diamagnetic nitrone or nitroso

*Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709.

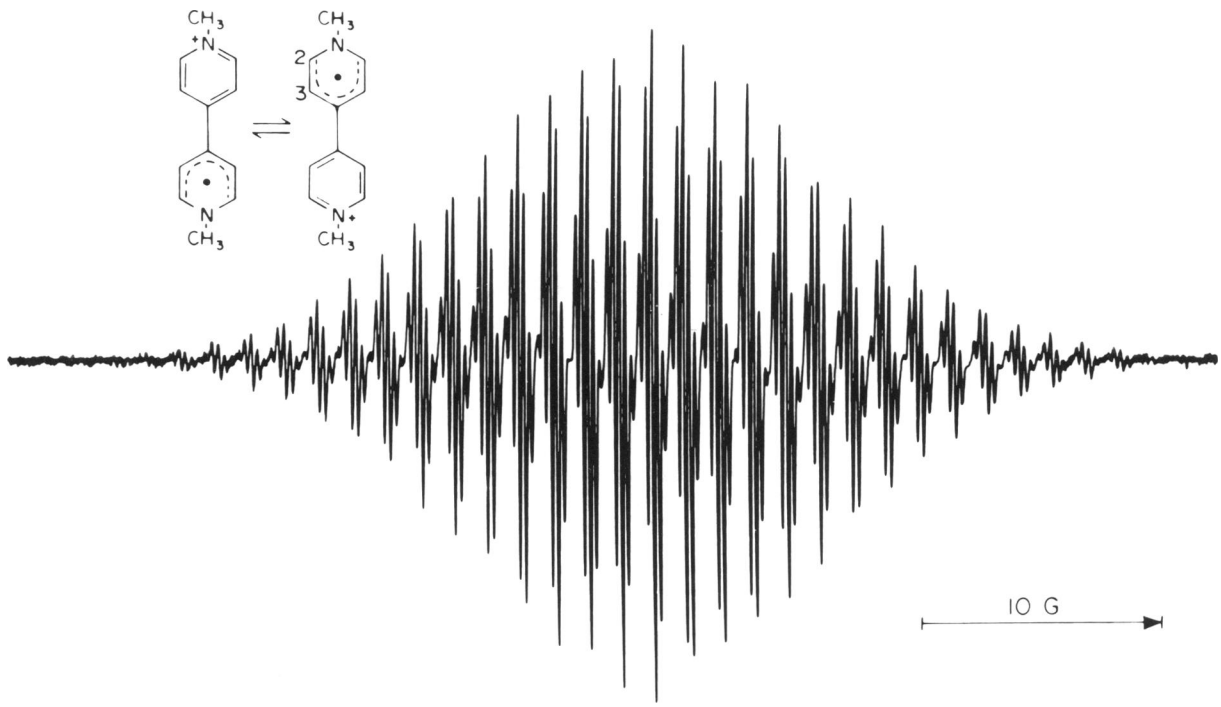


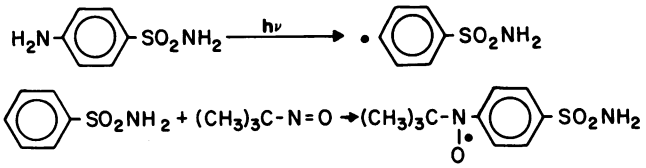
FIGURE 1. ESR spectrum of the paraquat cation free radical observed on incubation of 1 mM paraquat with 1 mg/mL of hepatic microsomes from male rats in KCl-Tris-$MgCl_2$ buffer (150 mM, 50 mM, and 5 mM, pH 7.4) containing 0.8 mM NADP+, 11 mM glucose 6-phosphate, and 1.3 units/mL of glucose-6-phosphate dehydrogenase. From Mason and Holtzman (*5*), with permission.

compound (spin trap) to form a more stable nitroxide free radical (spin adduct):

$$R\cdot + R'\text{—}N\text{=}O \rightarrow R'\text{—}\underset{\underset{O}{|\cdot}}{N}\text{—}R$$

$$R\cdot + R'\text{—}CH\text{=}\overset{+}{N}\text{—}R'' \rightarrow R'\text{—}\underset{R}{CH}\text{—}\underset{\underset{O}{|\cdot}}{N}\text{—}R''$$
$$\qquad\qquad\qquad\quad O^-$$

The structure of the parent free radical (R·) may then be determined from the hyperfine couplings of the ESR spectrum of the spin adduct. For example, the ESR spectrum of the radical formed during the ultraviolet irradiation of sulfanilamide in the presence of the spin trap 2-methyl-2-nitrosopropane is shown in Figure 2. This spectrum is easily identified as that of 4-*tert*-butylbenzenesulfonamide (*9*), which suggests the following mechanism for the photolysis:



A comprehensive review on the use of spin trapping to detect and identify free radical metabolites has recently appeared (*10*).

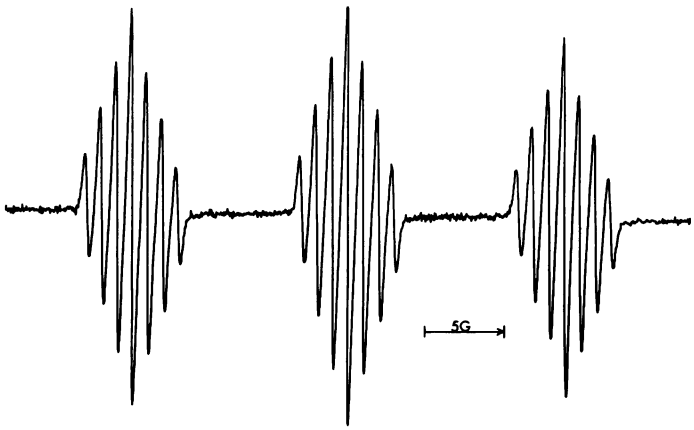


FIGURE 2. ESR spectrum of 4-sulfamoylphenyl-*t*-butylnitroxide formed during the UV irradiation of 4-aminobenzenesulfonamide (sulfanilamide) in the presence of MNP. Modified from Chignell et al. (*9*).

Free-Radical Pathways in Xenobiotic Metabolism

One-Electron Enzymatic Reduction of Xenobiotics

A wide variety of chemicals can be reduced to form free radicals by a number of different enzyme systems (Table 1). While oxygen has been omitted from Table 1 it should be pointed out that several of the enzymes will also reduce oxygen to its free radical form, superoxide

Table 1. Xenobiotics undergoing one-electron enzyme reduction.[a]

Compound class	Enzyme system[b]	Reference
Quinone	Nc,Nb,Cb,Nd,Er, Xd,Xo,Ld,Fe	(*3,4,11,12*)
Quinoneimine	Nc	(*4*)
Nitroaromatic	Nc,Nb,Nd,Er,Xo,Ao,Fe,Pf	(*3,4,33*)
Azoaromatic	Nc,Nb,Xo	(*3,4,34*)
Carbon tetrachloride	CP	(*3,4*)
Bipyridylium	Nc,CP,Er	(*3,4,12*)
Tetrazolium	Nc	(*3,4*)
Di-*N*-oxide	Er	(*4*)
Sulfur oxides	CP	(*2*)
Triphenylmethane dyes	CP,Nc	(*2*)

[a] Modified from Mason and Chignell (*2*).

[b] Enzyme abbreviations: Nc = NADPH-cytochrome c(P-450) reductase (NADPH); Cb = cytochrome b_5; CP = cytochrome P-450; Er = *Escherichia coli* reductase (NADPH); Xd = xanthine dehydrogenase (xanthine or NADH); Xo = xanthine oxidase (xanthine or NADH); Ld = lipoamide dehydrogenase (NADH); Ao = aldehyde oxidase (aldehyde); Fe = ferredoxin-NADP$^+$ reductase (NADPH); Nd = NADH-dehydrogenase (NADH); Pf = pyruvate ferredoxin oxidoreductase (pyruvate); Nb = NADH-cytochrome b_5 reductase.

($O_2^{\cdot-}$). From the data in Table 1 it is clear that aromatic molecules with vastly different structures may undergo one-electron reduction. Furthermore, some chemicals, e.g., nitroaromatic compounds and quinones, will accept an electron from almost any redox flavoprotein.

There have been relatively few studies that have attempted to relate structure to activity for compounds undergoing enzymatic one-electron reduction. Most of the work in this area has involved either the nitroaromatic compounds or the quinones.

Nitroaromatic Compounds. Aromatic nitrocompounds may be reduced in a series of steps to the corresponding amine:

$$R{-}NO_2 \xrightarrow{e^-} R{-}NO_2^{\cdot-} \xrightarrow[4H^+]{3e^-} R{-}NHOH \xrightarrow[H^+]{2e^-} R{-}NH_2$$

The one-electron reduction of a nitro compound gives rise to a nitro anion-free radical. Under aerobic conditions the nitro anion-radical reacts with oxygen to form superoxide with the regeneration of the parent nitro compounds:

$$R{-}NO_2^{\cdot-} + O_2 \rightarrow R{-}NO_2 + O_2^{\cdot-}$$

Aromatic nitro compounds, particularly those that contain a heterocyclic moiety, are widely used as chemotherapeutic agents in protozoal, fungal, and bacterial infections (*13*) and as radiosensitizers in cancer chemotherapy (*14*). These drugs are also known to be mutagenic and toxic to mammalian cells (*15*). The enzymatic formation of the nitro anion free radical is thought to play a key role in both the biological activity and toxicity of nitroaromatic compounds (*3,4*). Since the ease of formation of the nitro anion radical is related to the one-electron reduction potential (E_7^1) of the parent compound attempts have been made to correlate biological activity with this parameter (*15–20*).

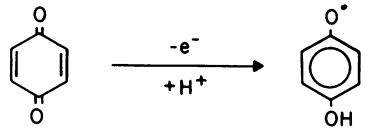
Adams and co-workers have tested 15 nitroaromatic and nitroheterocyclic compounds that can act as radiosensitizers for their toxicity to Chinese hamster V79 cells *in vitro* (*16*). They found that cytotoxicity increased markedly as the electron affinity, measured as a one-electron reduction potential, increased. A structure–activity relationship of the form

$$-\log C = b_0 + b_1 E_7^1 + b_2 \log P$$

was derived, where C is the specific cytotoxicity (concentration), E_7^1 is the one-electron reduction potential, P is the partition coefficient, and b_0, b_1, and b_2 are coefficients derived from the least-squares fit to the data. The potential E_7^1 is a measure of the ease of the one-electron reduction of a compound RNO_2 to the corresponding nitro anion-free radical $RNO_2^{\cdot-}$. Since the cells used in this study were hypoxic, it seems unlikely that the species involved in the toxic response is superoxide.

Adams and co-workers suggest instead that the four-electron reduction product of nitroaromatic compound, the corresponding hydroxylamine, may be the toxic species (*16*). Work by the same authors has shown that toxicity of nitroaromatic and nitroheterocyclic compounds towards the same cells under aerobic conditions is also related to their one-electron reduction potentials (*15*). However, in this case there was no correlation with octanol:water partition coefficient. It was found, however, that quinones having the same one-electron reduction potential as the nitroaromatic compounds exhibited similar toxicity. These findings suggest that under aerobic conditions the toxic species may indeed be superoxide.

Quinones. The one-electron reduction of quinones leads to the formation of the corresponding semiquinone radical:



This reaction may be catalyzed by a wide variety of enzymes (Table 1). For example semiquinone metabolites of anthracycline antibiotics have been detected by ESR in both anaerobic NADPH-microsomal incubations (*21–23*) and in Ehrlich ascites cells (*21*). Under aerobic conditions the semiquinone reacts with oxygen to give superoxide and regenerate the parent quinone (*24,25*) (Fig. 2). The generation of superoxide by the anthracycline antitumor agent adriamycin is thought to be responsible for the cardiotoxicity of this drug (*26*). Superoxide is known to give rise to other reactive oxygen species including hydrogen peroxide and the hydroxyl radical (*27*). The observation that vitamin E ameliorates adriamycin-induced cardiomyopathy (*28*) is consistent with the initiation of lipid peroxidation by oxygen radicals derived from the oxidation of the adriamycin semiquinone.

The relationship between the one-electron reduction potentials of quinones and their reduction by flavoproteins has been examined by Powis and his co-workers

Table 2. Metabolism of antitumor quinones by NADPH-cytochrome P-450 reductase.[a]

Quinone	E_7^1, mV	NADPH oxidation, μmole/min/mg
Control	—	0.00 ± 0.00
AZQ[b]	-168	3.51 ± 0.07
Adrenochrome	-253	2.29 ± 0.11
Mitomycin C	-271	0.93 ± 0.03
Adriamycin	-289	0.93 ± 0.02
Daunomycin	-305	0.60 ± 0.03
Aclacinomycin A	—[c]	0.20 ± 0.02
Anthracenedione	-348	0.06 ± 0.01

[a] Modified from Svingen and Powis (*29*).
[b] 2,5-diaziridinyl-3,6-bis(carbethoxy)amine-1,4-benzoquinone.
[c] Not known.

(*29–31*). Svingen and Powis have reported that the enzymic one-electron reduction of a series of quinone antitumor agents, including adriamycin, daunomycin and mitomycin C, by NADPH-cytochrome P-450 reductase increases at more positive values of E_7^1 (Table 2). Powis and Appel have studied the aerobic and anaerobic metabolism of a series of benzoquinones by three flavoenzymes that catalyze single-electron reduction (*30*). They found that metabolism was more closely related to single-electron reduction potential than the structural features or lipid solubilities of the benzoquinones. The lower limit for reduction by NADH:ubiquinone oxidoreductase with NADPH as the cofactor and purified NADPH-cytochrome P-450 reductase was a quinone single-electron reduction potential of −240 mV. However, the lower limit for quinone reduction with purified NADH-cytochrome b_5 reductase and NADH:ubiquinone oxidoreductase with NADH as a cofactor was a single electron reduction potential of 170 mV. In general it was found that more negative single-electron potentials resulted in decreased quinone metabolism.

Incubation of simple benzoquinones with isolated hepatocytes results in the liberation of superoxide (*31*) formed by the reaction of the corresponding semiquinone radicals with oxygen. Powis and co-workers have found that superoxide formation by simple benzoquinones or the anthracyclines is maximal at a quinone one-electron reduction potential of -70 mV. This pattern was qualitatively similar to that observed with mitochondrial NADH:ubiquinone oxidoreductase and microsomal NADH-cytochrome b_5 reductase. The observation that superoxide production by NADPH-cytochrome P-450 reductase is maximal at a quinone E_7^1 of -200 mV suggests that this enzyme is not rate-limiting for quinone-stimulated superoxide formation by hepatocytes.

One-Electron Enzymic Oxidation of Xenobiotics

Xenobiotics may be oxidized to free radicals by both peroxidases and oxidases (Table 3). Among the peroxidases, horseradish peroxidase (HPR) has received most attention. However, it seems likely that the mammalian peroxidases, e.g., myeloperoxidase, lactoperoxidase, and

Table 3. Xenobiotics undergoing one-electron oxidation.[a]

Compound class	Enzyme system	Reference
Hydroquinones	HRP,Cer,Lac	(*3,4,11*)
Hydroaminquinones	HRP,Cer	(*4*)
Aromatic amines	HRP,Cat,PGS,MetM, CP,Cer,Lac,Cyt	(*4*)
Phenothiazines	HRP,Cat,Cer	(*3,4*)
Hydroxyaromatics	HRP,MetH,Hem	(*3,4,11*)
Hydroxylamines	HRP,Lact,My,MetH,Hem	(*3,4*)
Hydrazines	HRP,PGS,Hem	(*2,3,4*)

[a] Modified from Mason and Chignell (*2*).
[b] Enzyme abbreviations: HRP = horseradish peroxiase (H_2O_2); Cat = catalase (H_2O_2 or ROOH); Lac = lactoperoxidase (H_2O_2); My = myeloperoxidase (H_2O_2); PGS = prostaglandin synthase (H_2O_2 or ROOH); MetM = metmyglobin (H_2O_2 or ROOH); MetH = methemoglobin (H_2O_2 or ROOH); CP = cytochrome P-450 (ROOH); Cer = ceruloplasmin (O_2); Hem = hemoglobin (O_2).

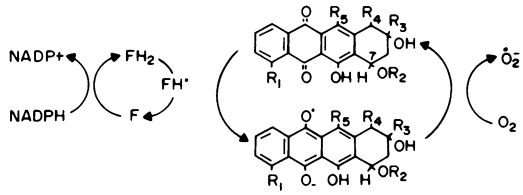


FIGURE 3. Mechanism of anthracycline-catalyzed superoxide anion radical generation. The exact scheme of electron donation by NADPH-cytochrome c reductase during semiquinone formation is unknown. From Mason (*3*) with permission.

prostaglandin synthase, probably catalyze many of the same reactions. It is interesting to note that peroxidase activity is also exhibited by catalase and methemoglobin. Among the oxidases that are known to generate free radicals, ceruloplasmin is the best studied.

There has been only one attempt to relate the one-electron enzymic oxidation of xenobiotics to structure. Job and Dunford have studied the effect of different substituents on the oxidation of phenols and aromatic amines by horseradish peroxidase (*32*). The one-electron oxidation of organic compounds (AH) by horseradish peroxidase may be depicted as follows:

$$\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I (green)}$$
$$\text{Compound I} + \text{AH}_2 \rightarrow \text{Compound II (red)} + \text{A}^{\cdot}\text{H}$$
$$\text{Compound II} + \text{AH}_2 \rightarrow \text{HRP} + \text{A}^{\cdot}\text{H}$$

Job and Dunford used stopped-flow spectrometry to measure the rate of reduction of HRP to compound I by a series of phenols and aromatic amines. They found that Hammett's rules apply for HRP Compound I reduction by monosubstituted phenols and anilines with different substituents in the *meta* or *para* positions. At pH 7.0, $\log k_X/k_H$ was found to be -6.92σ and -7.00σ for phenols and anilines, respectively, where k_H and k_X are the second-order rate constants for the reaction between compound I and an unsubstituted phenol or aromatic amine with the substituent X in the *meta* or *para* positions. This finding indicates that all phenols are oxidized by compound I by the same mechanism suggesting that in all cases the neutral forms of the phenols are

involved. The same conclusion can also be drawn for the substituted anilines. Since the *meta* and *para* substituents produce nearly the same effect, it follows that the rate-determining step in the oxidation of a phenol or an aromatic amine by compound I is the removal of one electron.

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